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Functional Expression of Two Arabidopsis Aldehyde Oxidases in the Yeast *Pichia pastoris*[1]

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To investigate the biochemical and enzymatic properties of two aldehyde oxidase (AO) isoforms of *Arabidopsis thaliana*, we expressed *AAO1* and *AAO2* cDNAs in a heterologous yeast (*Pichia pastoris*) system and successfully obtained the proteins in active forms. The expressed AAO1 and AAO2 proteins gave activity bands with the same mobilities on native gel electrophoresis and exhibited the same substrate preferences on zymograms with 8 aldehydes as those of AOα and AOγ in Arabidopsis seedlings, respectively. Furthermore, anti-AAO1 and anti-AAO2 antibodies, which specifically recognize the seedling AOα and AOγ, respectively, reacted with the AAO1 and AAO2 proteins produced in *P. pastoris*, respectively. These results indicate that these AO proteins are accurately produced in the yeast system, as in Arabidopsis seedlings. Using AO preparations from *P. pastoris*, the enzymatic properties of Arabidopsis AOα and AOγ were investigated. AOα showed a relatively wide substrate specificity for 7 aldehydes tested, with high affinity to benzaldehyde and indole-3-aldehyde, while AOγ could most efficiently oxidize naphthaldehyde. AOα was strongly inhibited by iodoacetate and KCN, while AOγ was inhibited not only by iodoacetate and KCN but also by 2-mercaptethanol, dithiothreitol, menadion, and estradiol. AOα and AOγ showed the highest activity at around 65 and 50°C, respectively, and exhibited pH dependence around pH 8.0. These results indicate that the two AO isoforms in Arabidopsis seedlings have different enzymatic properties and may have different physiological roles *in vivo*.

Key words: aldehyde oxidase, *Arabidopsis thaliana*, indole-3-acetic acid (IAA), molybdenum cofactor, *Pichia pastoris*.

Aldehyde oxidase (AO; aldehyde-oxygen oxidoreductase, EC 1.2.3.1) has been extensively investigated in animals and microorganisms, where it catalyzes the oxidation of a variety of aldehydes and *N*-heterocyclic compounds in the presence of O_2 or redox dyes. The enzyme is known to be a homodimer of about 150-kDa subunits, containing a molybdenum cofactor (Moco), nonheme iron and FAD as prosthetic groups (*1–3*). Animal AO is expressed at a high level in the liver and has been implicated in the detoxification of environmental pollutants and xenobiotics (*4*, *5*). The animal AO might also play a role in the conversion of retinal to retinoic acid (*6*).

In plants, the role of AO in the biosynthesis of plant hormones, such as indole-3-acetic acid (IAA) (*7–12*) and abscisic acid (ABA) (*13–18*), has been focused on, because the last step of these phytohormone biosynthesis pathways is oxidation of the corresponding aldehyde; indole-3-acetaldehyde (IAAld) for IAA and abscisic aldehyde for ABA. However, a plant AO has only been purified from maize coleoptiles (*10*), and molecular cloning of a plant AO has only been reported for maize, tomato and *Arabidopsis thaliana* (*19–21*). We still have a little information about the biochemical and molecular properties of plant AOs to discuss their functions *in vivo*.

Four independent cDNA clones (*AAO1*, *2*, *3*, and *4*, formerly called *atAO-1*, *-2*, *-3*, and *-4*) for putative Arabidopsis AOs have been cloned by our group (*21*) and three AO isoforms, AOα, AOβ, and AOγ, in Arabidopsis seedlings were detected on zymograms (*11*). Subsequently, we raised specific antibodies against partial peptides of the *AAO1* and *AAO2* products expressed in *Escherichia coli*, where the anti-AAO1 and anti-AAO2 antibodies recognized AOα and AOβ, and AOβ and AOγ, respectively. Furthermore, the partial amino acid sequences of polypeptides specifically recognized by these antibodies corresponded to those of the deduced amino acid sequences of the *AAO1* and *AAO2* products, respectively. We concluded that AOα and AOγ are homodimers consisting of the *AAO1* and *AAO2* products, respectively, and AOβ is a heterodimer of both products (*22*). In Arabidopsis seedlings these three isoforms showed different localization patterns; AOα is abundant in roots and AOγ is mainly detected in cotyledons. They also have different substrate preferences. These results indicate that

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Abbreviations: AO, aldehyde oxidase; DCIP, 2,6-dichloroindophenol; IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; Moco, molybdenum cofactor; mAMSA, 4′-(9-acridinylamino) methanesulfon-*m*-anisidine.

they have different physiological functions in Arabidopsis seedlings (*21*, *22*). However, because of the difficulty in obtaining a sufficient amount of an enzyme preparation for each AO from Arabidopsis seedlings, we only know their substrate preferences on zymograms after native PAGE.

For better understanding of their functions and properties, it is necessary to obtain each AO protein in an amount enough for biochemical and enzymatic studies in heterologous expression systems. In the present study, we developed a highly efficient functional expression system in the yeast *Pichia pastoris* for two Arabidopsis AO cDNAs, *AAO1* and *AAO2*, and using the AO preparations from yeast, we investigated their enzymatic and biochemical properties in detail.

MATERIALS AND METHODS

Construction of Expression Vectors—Standard molecular cloning techniques were used for DNA manipulation. *AAO1* and *AAO2* full length cDNAs (*21*) were cloned into pPICZC with an ACC sequence (*23*) just before the initiation ATG codon according to the instructions for an Invitrogen "Easy Select *Pichia* Expression Kit" (*24*). The constructs were named pPICAAO1 and pPICAAO2, respectively.

Expression of AAO cDNAs in P. pastoris—*P. pastoris* strain KM71 was transformed with 10 μg of linearized pPICAAO1, pPICAAO2, or pPICZC (vector only) by electroporation using a 2-mm electrode gap cuvette (Bio-Rad, Gene Pulser II). Transformants were selected on YPDS (1% yeast extract, 2% peptone, 2% glucose, and 1 M sorbitol) plates containing 100 μg/ml of zeocin. Zeocin-resistant colonies were checked for the introduction of the desired cDNAs by PCR, and several positive transformants were examined for the expression of AO activity in a small-scale incubation [50 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate, 4 × 10^{-5}% biotin, and 1% glycerol) in a 300-ml baffled flask]. The yeast cells were grown for 16–18 h at 30°C with shaking at 200 rpm and then collected by centrifugation. The pellet was resuspended in 15 ml of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate, 4 × 10^{-5}% biotin, and 0.5% methanol) containing 0.3 mM sodium molybdate in a 100-ml baffled flask and then cultured again at 30°C with shaking at 200 rpm. Methanol was added every 12 h to give a final concentration of 0.5%. After 0, 12, 24, 36, and 48 h of methanol induction, cells were collected and resuspended in a breaking buffer [50 mM sodium phosphate, pH 7.4, 1 mM EDTA, and 5% glycerol, containing protease inhibitors (Complete™ Protease Inhibitor cocktail tablets, Roche Diagnostics K.K.; one tablet/100 ml)]. After the cells had been broken by vigorous mixing with glass beads (450 to 600 μm, Sigma), a cell extract was obtained by centrifugation. The AO activity in the extract was examined by activity stain after native PAGE and the cell lines expressing high AO activity were selected by comparing the band intensities on the zymograms.

The AOs were then produced in a large-scale culture using the two selected transformed cell lines. The cells were first grown in a 1,000 ml baffled flask containing 200 ml of BMGY medium for 16 h with shaking at 200 rpm. The cells were collected by centrifugation and the pellet was resuspended in 50 ml of BMMY medium containing 0.3 mM sodium molybdate, and then the suspension was incubated in a 300-ml baffled flask for another 24 h. Methanol was added every 12 h to give a final concentration of 0.5%. The cells were collected by centrifugation and stored at −80°C until use. Cells were homogenized with the breaking buffer and glass beads in a Bead-Beater (Biospeck Products). The crude enzyme solution obtained was fractionated with ammonium sulfate (0–55% saturation), and then dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 1 μM sodium molybdate, 1 mM EDTA, 2 mM DTT, and the protease inhibitors. The supernatant was heat-treated (60°C, 3 min) to remove excess contaminating proteins. After centrifugation, the supernatant was used for further studies.

Native PAGE and Activity Stain—Native PAGE was performed on a 7.5% acrylamide gel at 4°C as described previously (*9*). After electrophoresis, the gel was immersed in 0.1 M potassium phosphate buffer (pH 7.5) for 2–3 min and then the activity band of AO was developed with a mixture comprising the 0.1 M potassium phosphate buffer (pH 7.5), 0.1 mM phenazine methosulfate, 0.4 mM MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazoliumbromide], and 1 mM substrate at 30°C in the dark for 30–60 min.

Immunoprecipitation—Polyclonal rabbit antibodies raised against about 30-kDa AAO1 and AAO2 peptides produced by *E. coli* were obtained by Akaba *et al.*, (1999) (*22*). The anti-AAO1 and anti-AAO2 peptide antibodies could specifically recognize AOα and AOγ in the Arabidopsis seedlings, respectively. Immunoprecipitation using these antibodies was performed with the AAO1 and AAO2 proteins produced in *P. pastoris*. The ammonium sulfate fractionated and heat-treated enzyme samples (about 50 μg of protein) were incubated with the antibodies (about 50 μg of protein) at 30°C for 1 h and then at 4°C overnight. A 10 μl bed volume of Protein A Sepharose (Protein A Sepharose CL-4B; Amersham Pharmacia Biotech.), which had been equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, was added to the mixture, followed by incubation at room temperature for 30 min, and then at 4°C for 60 min with occasional mixing. After centrifugation (10,000 ×*g*, for 20 min at 4°C), the AO activities in the supernatant were determined after native PAGE.

Enzyme Assay—Enzyme activity was assayed according to the method described previously (*9*). When aromatic aldehydes were used as substrates, the AO activity was determined as the amount of reaction product formed in the presence of O_2 as an electron acceptor. The reaction products derived from the aldehyde substrates (cinnamaldehyde, cinnamic acid; indole-3-aldehyde, indole-3-carboxylic acid; IAAld, IAA; benzaldehyde, benzoic acid; protocatechualdehyde, protocatechuic acid; naphthaldehyde, naphthoic acid) were detected and quantified with a reversed-phase HPLC.

The substrate specificity of AOs for aliphatic aldehyde was also measured by a photometric method with 2,6-dichloroindophenol (DCIP) as an electron acceptor. The reaction mixture (300 μl) contained 5 to 10 μl of enzyme solution, 0.1 M phosphate buffer (pH 7.5), 0.002% DCIP, 0.1 mM phenazine methosulfate, and the desired amount of substrate, and the decrease in A_{600} (16.1 mM^{-1} cm^{-1}) was monitored with a U-2000 double-beam photometer (Hitachi, Tokyo). The substrates were dissolved in 80% acetone and stocked in −20°C until used.

Chemicals—4′-(9-acridinylamino)methanesulfon-*m*-anisidine (mAMSA) was kindly supplied by the National Cancer Institute Chemotherapeutic Agents Repository (Maryland, USA). IAAld was prepared from IAAld bisulfite (Sigma) according to the method described by Bower *et al.* (1978) (7).

RESULTS

Heterologous Expression of AAO cDNAs—*AAO1* and *AAO2* full-length cDNAs were cloned into the pPICZC vector and the introduced into *P. pastoris* cells. The selection of transformant cells was performed on YPDS plates containing 100 μg/ml zeocin. We examined three *AAO1* and *AAO2* transformed cell lines for AO production after methanol induction using a small-scale system. During AO induction by methanol addition at 12-h intervals, the AO activity in the cell extracts was determined as the activity band on native PAGE with indole-3-aldehyde, naphthaldehyde, or a mixture of them as the substrate. An intense band for both *AAO1* and *AAO2* transformed cells was detected 12 h after the methanol induction and strong intensity was observed after 24 h. The activity then gradually decreased until 48 h of incubation. A typical result as to AO activity in the gel at 0 h and 24 h after methanol induction is shown in Fig. 1A. On SDS-PAGE, Coomassie Brilliant Blue–stained protein bands of 150 and 145 kDa were detected for transformed cells with pPICAAO1 and pPICAAO2, respectively, after methanol induction, whereas these protein bands were not detected for pPICZC-transformed cells (Fig. 1B). On immunoblot analysis it was also shown that these bands corresponded to *AAO1* and *AAO2* products, respectively (data not shown). The molecular masses of these bands were coincident with those estimated from the deduced amino acid sequences of *AAO1* and *AAO2*. From the band intensity, the yields of the expressed proteins were roughly estimated to be 10–15 mg from one liter culture of transformed *P. pastoris* cells.

Comparison of the Substrate Preference on Zymogram between Seedling AOs and AOs Expressed in P. pastoris—The zymogram patterns with 8 different aldehydes as substrates were compared between the seedling AOs and AOs produced in *P. pastoris* (Fig. 2). The mobilities of the activity bands of the *AAO1* and *AAO2* products in *P. pastoris* were identical to those of seedling AOα and AOγ, respectively. Intense bands were observed with indole-3-aldehyde and citral for seedling AOα, and a very similar zymogram

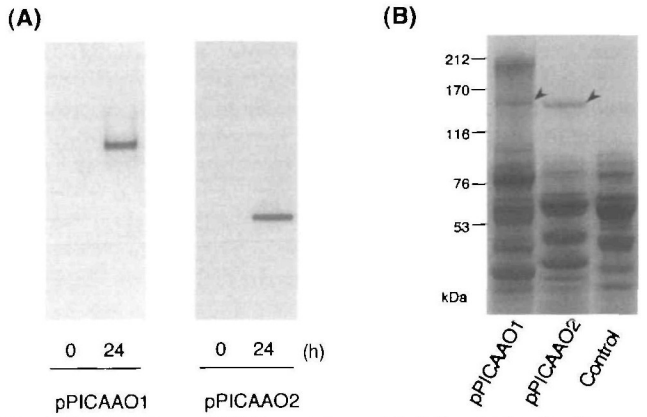

Fig. 1. **Production of AAO proteins with AO activity by *P. pastoris*.** (A) After 0 h and 24 h of methanol induction, the yeast cells transformed with pPICAAO1 and pPICAAO2 were collected and protein samples were prepared as described under "MATERIALS AND METHODS." After native PAGE, the AO activity in the cell extracts was developed using a mixture of indole-3-aldehyde and 1-napthaldehyde as substrates. (B) Protein samples from pPICAAO1, pPICAAO2, and pPICZC (control) transformed cells after 24 h of methanol induction were subjected to SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. Arrows indicate the protein bands corresponding to *AAO1* and *AAO2* products in pPICAAO1 and pPICAAO2 transformed cells, respectively.

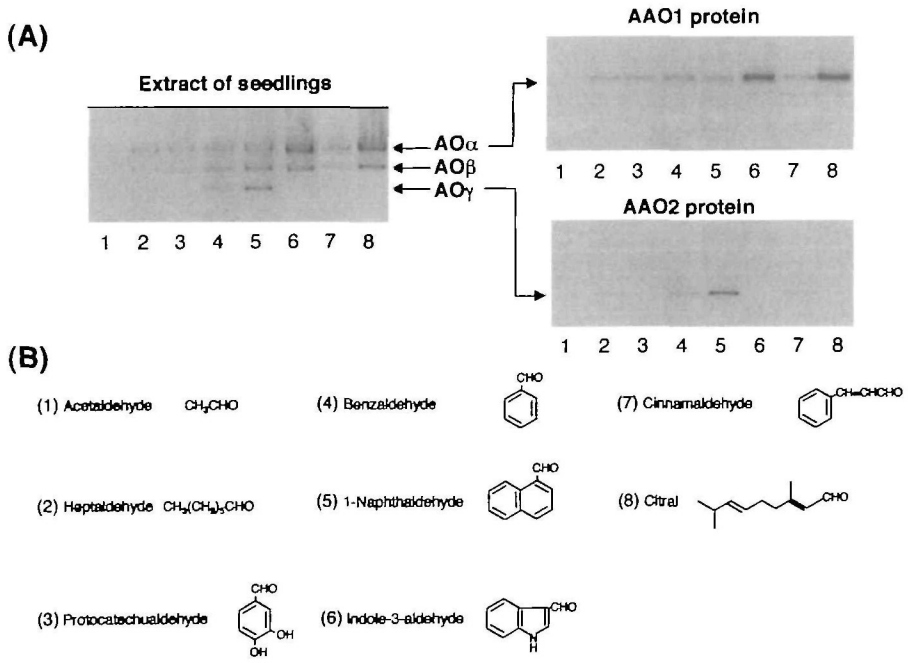

Fig. 2. **Zymograms of seedling AOs and AAO protein produced in *P. pastoris* with eight substrates.** Ammonium sulfate-fractionated and heat-treated enzyme samples obtained from the extracts of 8-day-old Arabidopsis seedlings and transformed *P. pastoris* cells were subjected to native PAGE. The activity bands were developed separately with strips of each lane using 8 aldehydes (A). The number at the bottom of each lane corresponds to the substrate indicated in (B).

pattern was obtained for the AAO1 protein. Seedling AOγ showed affinity for 1-naphthaldehyde, as *AAO2* product did. These results suggest that the substrate preferences of these AO proteins are practically identical to those of seedling AOs. We also determined their native-molecular masses by gel filtration chromatography. They were approximately 290–300 kDa (data not shown); these values were identical to those of seedling AOs (*21*). It can thus be concluded that in the *P. pastoris* system each enzyme was produced as a homodimer of 145–150 kDa subunits, which correspond to those calculated from the deduced amino acid sequences of *AAO1* and *AAO2*.

Identification of AAO Proteins with Specific Antibodies—Using specific antibodies against AAO1 and AAO2 (*22*), we examined whether or not the AOs expressed in *P. pastoris* could be specifically recognized by them (Fig. 3). Anti-AAO1 antibodies, which could specifically recognize seedling AOα, reacted with the *AAO1* product in *P. pastoris* but not with the *AAO2* product. While, anti-AAO2 antibodies, which exhibit immunoreactivity specific to seedling AOγ, did not cause any decrease in the enzyme activity of the *AAO1* product, but recognized the *AAO2* product. Based on the above results of native PAGE (see Fig. 2) and the molecular masses, we conclude that the AO proteins were produced in the heterologous *P. pastoris* system in exactly the same way as in Arabidopsis seedlings.

Enzymatic Properties of AOα and AOγ Produced in P. pastoris—Since we do not have purified plant AOα and AOγ from Arabidopsis, precise enzymatic characterization has not been performed yet. From the above results, we assumed that AOα and AOγ expressed in *P. pastoris* have the same enzymatic properties as those of plant enzymes, and some properties were investigated using AOα and AOγ produced in *P. pastoris*. The K_m values and specific activities of AOα and AOγ for 8 aldehydes, including IAAld, a possible precursor of IAA, are presented in Table I. AOα showed high affinity to benzaldehyde and indole-3-aldehyde, but could also oxidize four other aldehydes including IAAld; the K_m and specific-activity values were in the ranges of 0.7 to 39 μM and 3.8 to 38 nmol/min, respectively.

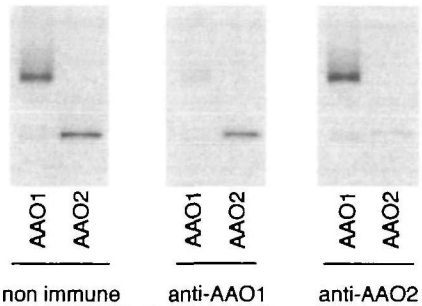

Fig. 3. **Immunoprecipitation of AAO proteins expressed in *P. pastoris* by specific antibodies.** Crude extracts of transformed *P. pastoris* cells were treated with two different rabbit antibodies [anti-AAO1 and anti-AAO2 antibodies (*22*)] and Protein A Sepharose as described under "MATERIALS AND METHODS." AO activity remaining in the supernatant was detected after native PAGE using a mixture of indole-3-aldehyde and 1-naphthaldehyde as substrates.

TABLE I. **K_m values and specific activities of Arabidopsis AOα and AOγ produced in *P. pastoris* for various substrates.**

Substrate	AOα		AOγ	
	K_m (μM)	Specific activity[c]	K_m (μM)	Specific activity[c]
Heptaldehyde[a]	14	7.1	57	24
Protocatechualdehyde[b]	19	8.0	—	—
Benzaldehyde[b]	0.74	17	7.7	8.7
Naphthaldehyde[b]	—[d]	—	0.33	65
Indole-3-aldehyde[b]	4.4	6.9	—	—
Indole-3-acetaldehyde[b]	39	7.3	—	—
Cinnamaldehyde[a]	20	3.8	410	20
Citral[a]	22	38	—	—

Crude enzyme solutions were obtained from *P. pastoris* cells transformed with *AAO1* and *AAO2* cDNA, respectively. The ammonium sulfate-fractionated and heat-treated samples were used for determination of the AO activity. [a]The enzyme activity was assayed by monitoring the reduction of DCIP as an electron acceptor. [b]The enzyme activity was assayed by determining the amount of the reaction product formed in the presence of O_2 as an electron acceptor. [c]Nanomoles of product formed or DCIP reduced min^{-1} mg^{-1} protein. [d]Not determined.

TABLE II. **Effects of the agents on Arabidopsis AOα and AOγ produced in *P. pastoris*.**

Agent	Concentration (mM)	Activity (%)	
		AOα	AOγ
Control		100	100
2-Mercaptoethanol	5.0	89	21
DTT	5.0	82	20
p-Chloromercuribenzoate	0.05	100	18
Iodoacetate	5.0	5.3	0
KCN	1.0	<5	13
Menadione	0.1	85	1.7
Estradiol	0.1	72	17
MAMSA	0.01	82	76
Allopurinol	0.25	76	110
TritonX-100	0.01%[a]	89	75

Crude enzyme solutions were obtained from *P. pastoris* cells transformed with *AAO1* and *AAO2* cDNA, respectively. The ammonium sulfate-fractionated and heat-treated samples were used for determination of the AO activity. The activity was assayed by using HPLC to determine the amounts of indole-3-carboxylic acid (for AOα) and benzoic acid (for AOγ) formed from indole-3-aldehyde and benzaldehyde, respectively. The enzymes were preincubated with the agent for 3 min before the reaction was started. [a](v/v).

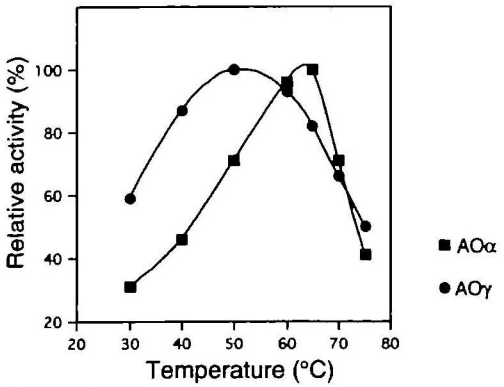

Fig. 4. **Effects of the reaction temperature on the activities of Arabidopsis AOα and AOγ expressed in *P. pastoris*.** AO activity was assayed at the temperatures indicated by HPLC to determine the amounts of indole-3-carboxylic acid (for AOα) and benzoic acid (for AOγ) formed from indole-3-aldehyde and benzaldehyde, respectively. AO activities are presented as activity relative to the maximum activity. The presented figure is a typical result for three independent experiments.

However, oxidizing activity toward naphthaldehyde could not be detected. On the other hand, AOγ showed a rather narrow substrate preference, naphthaldehyde being most efficiently oxidized with a low K_m value of 0.33 μM. The enzyme could not use IAAld as a substrate.

Several agents were examined for their effects on the AOα and AOγ activities (Table II). Iodoacetate and KCN had strong inhibitory effects on both AOs. AOγ was affected by 2-mercaptethnol and dithiothreitol, the activity being reduced to 21 and 20%, respectively. Moreover, menadion and estradiol, which are known to be strong inhibitors of animal AO (*25–27*), showed strong inhibition of the AOγ activity to 1.7 and 17%, respectively. However, mAMSA, a potent inhibitor of animal AO, had only slight effects on both AOs. Allopurinol, an inhibitor of xanthine dehydrogenase/oxidase, had no effect on these AO activities, indicating that these enzymes are distinguishable from xanthine dehydrogenase/oxidase.

Both AOs exhibited a pH optimum of around pH 8.0. AOα and AOγ showed the maximum activity around 65 and 50°C, respectively (Fig. 4).

DISCUSSION

Heterologous expression systems for heme-containing Moco-enzymes, such as plant nitrate reductase in methylotrophic yeast *P. pastoris* (*29–31*), have been reported, even with mutated recombinant proteins for analysis of the functional domain and/or the site of regulation of the activity. However, functional expression of a nonheme iron (iron-sulfur)-containing Moco-enzyme, AO and xanthine dehydrogenase/oxidase from animal and plant sources in heterologous systems was only reported very recently for mouse AO/retinal oxidase, with an *E. coli* system (*32*). This seems to be due to not only their requirement of complex prosthetic groups, Moco, FAD and nonheme iron, but also their large molecular weight (dimer of around 150,000 MW subunits).

We obtained four different AO genes from Arabidopsis (*AAO1*, *AAO2*, *AAO3*, and *AAO4*), and we detected several AO isoforms on zymogram in this plant. So an efficient heterologous expression system has been desired to produce an active enzyme for each gene to determine which isoform its product corresponds to, and to characterize its enzymatic and molecular properties. By means of immunological methods, we previously suggested that two *AAO* genes, *AAO1* and *AAO2*, encode three AO isoforms in Arabidopsis seedlings (AOα, AOβ, and AOγ), where AOα and AOγ are homodimers composed of *AAO1* and *AAO2* products, respectively, and AOβ is a heterodimer of them (*22*). However, characterization of each isoform could not be performed without separating them from the seedling extracts. In the present study, we succeeded in efficiently expressing these gene products in a *P. pastoris* system, in which the AAO1 and AAO2 proteins have exactly the same enzymatic properties as those of seedling AOα and AOγ, respectively; with respect to the mobility on native PAGE, the zymogram pattern with 8 aldehydes, the immunological reactivity and the molecular mass. These data clearly indicate that the *AAO1* and *AAO2* genes encode seedling AOα and AOγ, respectively, confirming our previous immunological identification (*22*). Since we proposed that AOβ is a heterodimer of the *AAO1* and *AAO2* proteins, we tried to express both the *AAO1* and *AAO2* cDNA in the same *P. pastoris* cells. However, in the transformed cells AOα and AOγ were produced, but AOβ could not detected (data not shown). It might be that some specific factor(s) is required for the assembly of a heterodimer from different subunits.

Using a *P. pastoris* system, we successfully produced sufficient amounts of Arabidopsis AOα and AOγ to investigate their biochemical and enzymatic properties. In our previous studies, purified maize coleoptile AO (*10*) and partially purified tobacco leaf AO (*17*) were shown to exhibit relatively broad substrate specificities. In *A. thaliana* we also reported that three isoforms, AOα, AOβ, and AOγ, in seedlings showed different but wide substrate specificities with a semi-quantitative method of activity stain after native PAGE (*11*). In the present study, we could directly assay AOα and AOγ activity using enzyme preparations produced with *P. pastoris* cells. AOα exhibited a relatively broad substrate specificity, while AOγ showed a preference for 1-naphthaldehyde. A remarkable difference was also observed in the perception of several inhibitors between AOα and AOγ. These results and the previous observation showing different tissue distributions (*11*, *22*) indicate that AOα and AOγ in Arabidopsis seedlings have different physiological roles in this plant.

Among three Arabidopsis AOs, maize coleoptile AO (*10*) and *Nicotiana plumbaginifolia* leaf AO (*17*), Arabidopsis AOγ showed some notable differences in enzymatic properties, especially in the sensitivity to common inhibitors for animal AOs, menadione and estradiol. This is the first suggestion of the possibility of the presence of an animal-like AO in plants, but the enzyme could not oxidize N^1-methylnicotinamide, one of the most efficient substrates for animal AOs (data not shown). In addition, since no high sequence homology between plant and animal AO DNAs was found (*19*), it is still difficult to discuss the similarity between plant and animal AOs. Based on present results, it can be mentioned that Arabidopsis AOα, maize coleoptile AO and *N. plumbaginifolia* leaf AO exhibit some similarity in their natures.

In our previous study involving maize coleoptiles, we proposed that an AO purified using IAAld as a substrate was a possible candidate of the enzyme which is involved in the biosynthesis of plant hormone IAA, because of the low K_m value for IAAld, a possible precursor of IAA, and its localization in the apical region of coleoptiles, a putative site of IAA biosynthesis (*10*). Studies involving an Arabidopsis IAA-overproducing mutant, *superroot1* (*sur1*), revealed that AOα can efficiently oxidize IAAld, and that the activity was significantly higher in *sur1* seedlings than in wild-type seedlings (*11*), suggesting the possible involvement of AOα in IAA biosynthesis. The present results indicate that AOα exhibits a relatively wide substrate specificity including IAAld, as observed for maize AO. Although there was some difference in the K_m values for IAAld; 3.2 and 39 μmol of maize AO (*10*) and Arabidopsis AOα, respectively, the values could explain a function of these AOs in IAA biosynthesis *in vivo*.

In the present work, because we used partially purified AO preparations derived from transformed *P. pastoris* cells, accurate investigation of enzymatic properties, such as kinetic parameters (K_m, V_{max}) and inhibition kinetics for some inhibitors, using purified AOs, remains to be performed. For this purpose, we are now trying to express the polyhis-

tidin-tagged AO proteins in the yeast system. This will also be useful for determining the fine protein structure, including the Moco nature (what type of Moco), and domain structures for the catalytic activity and its regulation, by utilizing mutated recombinant AO proteins. To investigate their physiological role in Arabidopsis plants, we are also conducting experiments with transgenic Arabidopsis with sense and antisense *AAO* cDNAs and knockout Arabidopsis lines (T-DNA insertion lines) for each *AAO* gene.

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